8 The genus *Bifidobacterium*

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8.1 General description of the genus

The first recorded mention in the annals of science of the name '*bifidus*' as applied to a cell dates to 1900, when Tissier (1900) discovered in the faeces of breast-fed infants a rod-shaped, Gram-positive, non-gas-producing, anaerobic bacterium with bifid morphology which he termed *Bacillus bifidus*. At the beginning of the 20th century, Orla-Jensen, in a detailed paper on bacteria that produce lactic acid, classified the *Bacillus bifidus* as part of the Lactobacteriaceae family, and in 1924 he tried to propose it as a separate species, explaining that the various species of bifidobacteria "doubtless constitute a separate genus, possibly forming a connecting link between lactic acid bacteria and the propionic acid bacteria". Although studies of this bacterial group gradually declined therafter, since 1950 there has been a flourish of new research that has brought the initial listing of *Lactobacillus bifidus* in the seventh edition of Bergey's *Manual of Determinative Bacteriology* (Breed *et al.*, 1957) to the present 24 species noted in its latest edition (Scardovi, 1986). Since then an additional five species have been described (*B. gallicum*, Lauer, 1990; *B. gallinarum*, Watabe *et al.*, 1983; *B. ruminantium* and *B. merycicum*, Biavati and Mattarelli, 1991; *B. saeculare*, Biavati *et al.*, 1991a).

One of the initial studies that led to the separation of bifidobacteria from lactobacilli was a 1957 paper by Dehnert, who proposed dividing bifidobacteria into five groups on the basis of their sugar fermentation: either pentose negative or pentose positive. A more detailed study was published in 1963 by Reuter, who isolated seven new species of bifidobacteria in the faeces of children, adults and adolescents on the basis of fermentation and serological characteristics: *B. infantis*, *B. parvulorum*, *B. breve*, *B. liberorum*, *B. lactentis*, *B. adolescentis* and *B. longum*. Confirmation of Reuter's work was provided in 1969 by the Japanese researcher Mitsuoka, who added two new biotypes to the *B. longum* species (*B. longum* subsp. *animalis a* and *b*) and isolated for the first time from the faeces of pig, chicken, calf and rat the two new species *B. thermophilum* and *B. pseudolongum*. Simultaneously in Italy Scardovi *et al.* (1969) isolated *B. ruminale* and *B. globosum* from the rumen of cattle and *B. asteroides*, *B. indicum* and *B. coryneforme* from the intestine of the honey bee.

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The species described to date can be regrouped in four different ecological niches: the human intestine, vagina and oral cavity, the animal intestine, the insect intestine and sewage. The intestinal microflora, one of the most complex ecological niches, is still not fully understood. Many types of facultative anaerobic bacteria live together, influencing each other. Host development age, the differing kinds of nourishment and the adaptability of each type influence the composition of microflora in the intestine, which can thus be considered as one of the most complex and variable of habitats. Only after the first week of life, or even later, is a comparatively permanent presence of microflora in the colon established.

While in the first day of life the faeces of both bottle- and breast-fed infants contain several facultative type groups, it takes another 5–7 days for flora bifida to be established: the bifidobacteria become the predominant bacteria in the faeces of breast-fed infants (Mitsuoka *et al.*, 1973; Biavati *et al.*, 1984) whereas no such predominance has ever been noted in the faeces of bottle-fed infants. This compositional diversity of the microflora in infants nourished with mother's or artificial milk is due to the fact that the former has specific growth factors for the development of bifidobacteria: for the *B. bifidum* var. *pennsylvanicus* the growth factor is an oligosaccharide mixture containing *N*-acetylglucosamine, glucose, galactose and fructose (Gyorgy, 1953; Gauche *et al.*, 1954). In the adult intestine the most frequently found bifid species are *B. pseudocatenulatum* and *B. longum* (Biavati *et al.*, 1986). Bifidobacteria have also been located in the oral cavity; the most common is *B. dentium* (Scardovi and Crociani, 1974), which seems to be involved in dental plaque formation.

Many types of bifidobacteria have been found in animal faeces (rabbit, chicken, cattle, mice and piglets), some of which seem to be 'host specific': *B. magnum* and *B. cuniculi* only in rabbit faeces, *B. pullorum* and *B. gallinarum* in the intestine of chicken, and *B. suis* only in piglet faeces. From a phylogenetically antique ecological niche two species have been isolated: *B. asteroides* is the only species found in *Apis mellifera* intestine and *B. indicum* in that of *Apis cerana* and *Apis dorsata* (from the Philippines and Malaysia) (Scardovi and Trovatelli, 1969). While in the early years of research it was widely believed that the bifidobacteria were typically confined to the ecological nich of the intestine, twelve species of this genus have been found in sewage, two of them, *B. minimum* and *B. subtile*, being found nowhere else (Biavati *et al.*, 1982): a fact that might be a sign of faecal pollution of water.

Many media have been studied for the isolation and the growth of bifidobacteria, essential components including sheep or horse blood (Dehnert, 1957; Reuter, 1963; Mitsuoka *et al.*, 1965), human milk (Dehnert, 1957), trypticase (BBL) and proteose peptone No. 3 (DIFCO), phytone (BBL), yeast extract (DIFCO), carbon source, mineral salts like Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, and vitamins like biotin and calcium

pantothenate. One of the most frequently used media for isolation is TPY (Scardovi, 1986): 10 g trypticase (BBL); 5 g phytone (BBL); 15 g glucose; 2.5 g yeast extract (DIFCO); 1 ml Tween 80; 0.5 g cystein hydrochloride; 2 g K_2HPO_4; 0.5 g $MgCl_2.6H_2O$; 0.25 g $ZnSO_4 \cdot 7H_2O$; 0.15 g $CaCl_2$; $FeCl_3$ trace; and 15 g agar to 1 litre water at pH 6.5. Since on this non-selective medium other bacteria can develop, such as streptococci and lactobacilli, whose colony morphology is indistinguishable from that of bifidobacteria, it is necessary to isolate on selective ground. Among the latest media with a considerable degree of selectivity are those of Muñoa and Pares (1988) and Beerens (1990).

Bifidobacteria have cell, bifid or multiple-branching rods and exhibit pleomorphism: in adverse growth conditions its morphology undergoes changes resulting in a cell with greater branching. In a medium deficient in ß-methyl-D-glucosamine the bifid cell takes on a more 'branched shape' (Glick *et al.*, 1960), and by adding certain amino acids (e.g. serine, alanine, aspartic acid) to a medium minimum and highly branched bifida cells become curved rods. The genus *Bifidobacterium* can generally be characterized as gram-positive, non-spore-forming, non-motile anaerobes (some species can tolerate O_2 only in the presence of CO_2) that are catalase-negative (excepting *B. asteroides* and *B. indicum* which are catalase positive when grown in the presence of air with or without the addition of hemin) and saccharoclastic (acetic and lactic acids are formed in the molar ratio of 3:2 without CO_2 production except in the degradation of gluconate). Small amounts of formic acid, ethanol and succinic acid are produced, while butyric and propionic acid never are. Glucose is degraded exclusively and characteristically by fructose-6-phosphoketolase (F6PPK-EC 4.1.2.22), which cleaves fructose 6-phosphate into acetylphosphate and erythose 4-phosphate. The G+C content of DNA (Bd or Tm) varies from 55–67 mol%.

Like all the Gram-positive bacteria, the cell wall of bifidobacteria consists of three macromolecular components: peptidoglycan (murein), polysaccharides and lipoteichoic acid. Kandler and Lauer (1974) and Lauer and Kandler (1983), in detailed studies on the peptidoglycan structures, showed that peptidoglycan is made up of *N*-acetylmuramic acid and *N*-acetylglucosamine, which form a polymer bound via oligopeptide chains whose interpeptide bridges differ from species to species, and proposed them as taxonomic characteristics. The same authors also report that the cell wall contains signficiant amounts of polysaccharides which usually consist of glucose and galactose and are often associated with rhamnose.

Two research groups, one from The Netherlands and the other from Germany, have taken an interest in the structure of the lipoteichoic acid, leading to controversial results. Veerkamp *et al.* (1983) in The Netherlands classified it chemically as the 1,2-linked phosphoglycerol oligomer containing either a galactose or glucose polymer chain at its hydrophilic

end and the glycerol 1-phosphoryl-diacyl-galactofuranosyl-diacylglyceride component at its hydrophobic end. For Fischer (1987) in Germany the chemical structure of the lipotheicoic acid is a galactofuranose-gluco-pyranose polymer, with glycerophosphate residues attached to the galactose residues.

The bifidobacteria ferment various types of sugars. Some, like lactose, galactose and sucrose, are metabolized by a large number of species while others, like mannitol or sorbitol, are fermented by a restricted number of species. The fermentative characteristics of the species of the genus *Bifidobacterium* are reported in Table 8.1. Bifidobacteria also have extracellular or surface enzymes that catalyse the hydrolysis of complex polysaccharides: amylose, amylopectin, xylan, gum arabic (Salyers *et al.*, 1978); the gastric mucin of pigs is degraded in the species *B. bifidum*, *B. infantis* and *B. longum* (Hoskins *et al.*, 1985) by enzymes that are partially extracellular and partially associated with the cells; dextran is metabolized by a dextranase that seems to be an extracellular endolytic enzyme (α-1,6 glucosidase).

In 1965 Scardovi and Trovatelli were the first to discover the 'fructose-6-phosphate shunt' (Figure 8.1). This pathway, often referred to as the bifid pathway, is utilized by many authors as a marker for the genus *Bifidobacterium*. The key enzyme of this pathway is a fructose-6-phosphate phosphoketolase which splits the hexose phosphate to erythrose-4-phosphate and acetyl phosphate. From tetrose and hexose phosphates, through the subsequent action of transaldolase and transketolase, are formed pentose phosphates that, via the usual 2–3 cleavage, give rise to lactic acid and additional amounts of acetic acid so that lactic and acetic acids are formed in the theoretical ratio 1.0:1.5. Phosphoroclastic cleavage of some pyruvate to formic and acetic acids and the reduction of acetate to ethanol can often alter the fermentation balance (De Vries and Stouthamer, 1968) to a highly variable extent (Lauer and Kandler, 1976).

The enzyme fructose-6-phosphate phosphoketolase, the key enzyme of glycolytic fermentation, also serves as a taxonomic character in the identification of the genus but does not enable the distinguishing of one species from another. In order to identify one species from another biochemically, electrophoretic studies were carried out on the transaldolase enzymes (EC2.2.I.2) and 6-phosphogluconate dehydrogenase (ECI.I.I. 44) (Scardovi *et al.*, 1979a). Fourteen isoenzymes of transaldolase and nineteen 6PGD have been identified: their distribution among the various bifidobacterial species is shown in Table 8.2. The urease activity found in nearly all the strains of the species *B. suis* and in a few strains of *B. breve*, *B. magnum* and *B. subtile*, strains grown in the absence of the urea, forces us to consider that this enzyme can not be induced (Craciani and Matteuzzi, 1982). The enzymes glutamate dehydrogenase and glutamine synthetase, which are involved in the assimilation of ammonia, were found

Figure 8.1 The fermentative characteristics of the species of the genus *Bifidobacterium**

	Sorbitol	L-Arabinose	Raffinose	D-Ribose	Starch	Lactose	Inulin	Cellobiose	Melezitose	Gluconate	Xylose	Mannose	Fructose	Galactose	Sucrose	Maltose	Threalose	Meliniose	Mannitol	Salicin
1. *B. bifidum*	−	−	−	−	−	+	−	−	−	−	−	−	+	+	d	−	−	±	−	−
2. *B. longum*	−	+	+	+	−	+	−	−	+	−	d	d	+	+	+	+	−	+	−	−
3. *B. infantis*	−	−	+	+	−	+	d	−	−	−	d	d	+	+	+	+	−	+	−	−
4. *B. breve*	d	−	+	+	−	+	d	d	d	−	−	+	+	+	+	+	d	−	d	+
5. *B. adolescentis*	d	+	+	+	+	+	d	+	+	+	+	d	+	+	+	+	d	−	d	+
6. *B. angulatum*	d	+	+	+	+	+	+	−	−	d	+	−	+	+	+	+	−	−	−	−
7. *B. catenulatum*	+	+	+	+	−	+	d	+	−	d	+	−	+	+	+	+	d	+	d	−
8. *B. pseudocatenulatum*	d	+	+	+	+	+	−	d	−	d	+	+	+	+	+	+	d	−	+	−
9. *B. dentium*	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+	−
10. *B. globosom*	−	d	+	+	+	+	−	−	−	−	d	−	+	+	+	+	−	+	−	−
11. *B. pseudolongum*	−	+	+	+	+	d	−	d	d	−	+	+	+	+	+	+	−	+	−	−
12. *B. cuniculi*	−	+	−	−	+	−	−	−	−	−	+	−	−	+	+	+	−	+	−	−
13. *B. choerinum*	−	−	+	−	+	+	−	−	−	−	−	−	−	+	+	+	−	+	−	−
14. *B. animalis*	−	+	+	+	+	+	−	d	d	−	+	d	+	+	+	+	d	+	−	+
15. *B. thermophilum*	−	−	+	−	+	d	d	d	d	−	−	−	+	+	+	+	d	+	−	d
16. *B. boum*	−	−	+	−	+	d	+	−	−	−	−	−	+	+	+	+	−	+	−	−
17. *B. magnum*	−	+	+	+	−	+	−	−	−	−	+	−	+	+	+	+	−	+	−	−
18. *B. pullorum*	−	+	+	+	−	−	+	−	−	−	+	+	+	+	+	+	+	+	−	+
19. *B. gallinarum*	−	+	+	+	−	+	+	d	d	ND	+	d	+	+	+	+	+	+	−	+
20. *B. suis*	−	+	+	−	−	+	−	−	−	−	+	d	d	+	+	+	−	+	−	−
21. *B. minimum*	−	−	−	−	+	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−
22. *B. subtile*	+	−	+	+	+	−	d	−	+	+	−	−	+	+	+	+	d	+	−	d
23. *B. coryneforme*	−	−	+	+	−	−	ND	+	−	+	+	−	+	ND	+	+	−	+	−	+
24. *B. asteroides*	−	−	+	+	−	−	−	+	−	d	+	−	+	d	+	d	−	+	−	+
25. *B. indicum*	−	−	+	+	−	−	−	+	−	+	−	d	+	d	+	d	−	+	−	+
26. *B. gallicum*	−	+	−	+	+	−	−	−	−	−	+	−	+	+	+	+	−	−	−	+
27. *B. ruminantium*	−	−	+	+	+	+	−	−	−	−	−	−	+	+	+	+	−	−	+	+
28. *B. merycicum*	−	+	+	+	+	+	∓	∓	−	−	+	−	∓	+	+	+	−	−	−	∓
29. *B. seaculare*	−	+	+	+	−	∓	+	−	+	−	+	+	+	∓	+	+	+	+	−	−

*Symbols: d, 11–89% of strains are positive; ND, not determined; ∓, when positive it is weakly fermented.

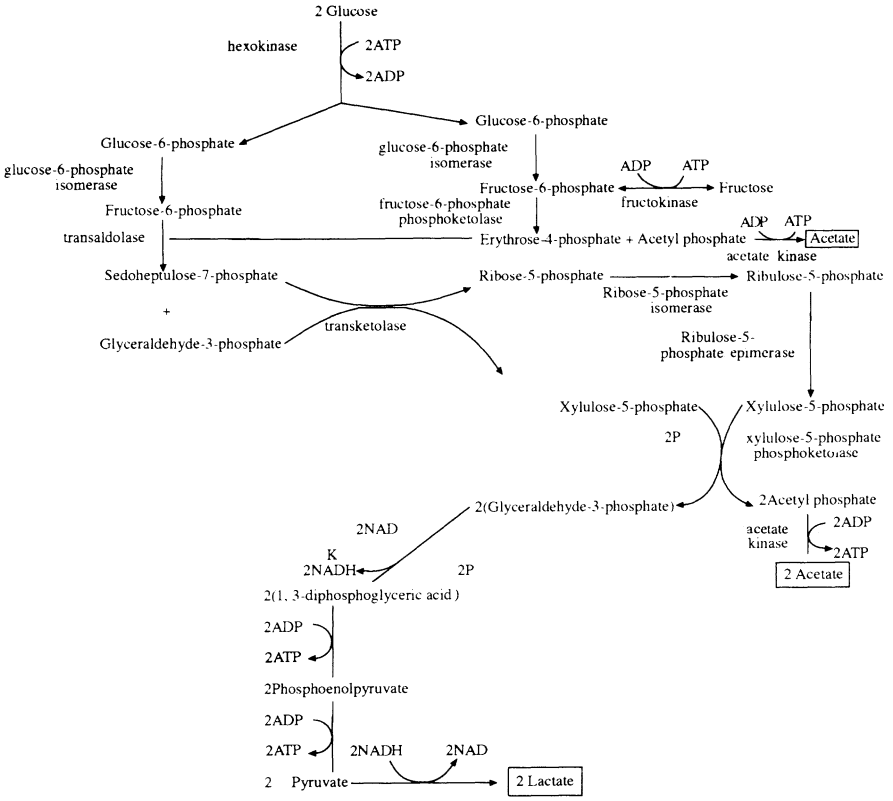

Figure 8.1 The fructose-6-phosphate shunt.

only in the species *B. bifidum*, *B. infantis*, *B. breve*, *B. adolescentis*, *B. thermophilum*, *B. longum* and *B. pseudolongum* (Hatanaka *et al.*, 1987).

The research carried out on the secretion activity of the bifidobacteria has highlighted a production of amino acids, the most frequent being alanine, valine and aspartic acid in strains of the species *B. thermophilum*, *B. adolescentis*, *B. dentium*, *B. animalis* and *B. infantis*. Exceptionally, *B. bifidum* can produce up to 150 mg/litre of threonine (Matteuzzi *et al.*, 1978). In 1977 Crociani *et al.* pointed out that the production of isoleucine and valine in mutants of *B. thermophilum* increased when the cell was grown in the presence of aminoisobutyric acid, a precursor of isoleucine.

Molecular-structure studies were initiated by Scardovi *et al.* (1971). Methodologies of DNA–DNA hybridization and the value of mol% G+C have reconfirmed species that had initially been proposed only on the basis of phenotypic characteristics (see Table 8.2). Subsequently, a total of 1500 bacteria isolates representing all the species of the genus were examined for the presence of plasmids. Approximately 20% contained detectable

Table 8.2 Mol% G+C values and electrophoretic patterns of transaldolase and 6-phosphogluconate dehydrogenase enzymes*

Species	% G+C (average values)	6-PGD isoenzyme	Transaldolase isoenzyme
1. *B. bifidum*	60.8	7	7
2. *B. longum*	60.8	5	6–8
3. *B. infantis*	60.5	4	3
4. *B. breve*	58.4	6–7	6
5. *B. adolescentis*	58.9	5	8
6. *B. angulatum*	59.0	5	5
7. *B. catenulatum*	54.0	6–8	5
8. *B. pseudocatenulatum*	57.5	1–3	4
9. *B. dentium*	61.2	–	4
10. *B. globosom*	63.8	6	2
11. *B. pseudolongum*	59.5	7	2
12. *B. cuniculi*	64.1	4	1
13. *B. choerinum*	66.3	4	3
14. *B. animalis*	60.0	8–9	5
15. *B. thermophilum*	60.0	7–8–9	8
16. *B. boum*	60.0	8–9	6
17. *B. magnum*	60.0	7	5
18. *B. pullorum*	67.5	Absent	2
19. *B. gallinarum*	65.7	Absent	Present
20. *B. suis*	62.0	5–8	6
21. *B. minimum*	61.5	6	10
22. *B. subtile*	61.5	2	3
23. *B. coryneforme*	—	6	6
24. *B. asteroides*	59.0	10	8
25. *B. indicum*	60.0	6–8	7–8–9
26. *B. gallicum*	61.0	ND	ND
27. *B. ruminantium*	57.0	Present	Present
28. *B. merycicum*	59.0	Present	Present
29. *B. saeculare*	63.0	Absent	Present

*Symbols: ND, not determined. Present, determined by other method.

plasmids but only four species were represented: *B. longum*, *B. globosum*, *B. asteroides* and *B. indicum*. The plasmid profile is multiple in *B. longum* and *B. asteroides* while the strains of the species *B. globosum* and *B. indicum* exhibit only one profile (Sgorbati *et al.*, 1982).

8.2 Phylogenetic relationships

The identification of bifidobacteria as a phylogenetically separate type and its relatedness to similar bacterial types have been the subject of studies and controversies for years. Indeed, bacterial phylogeny is no simple matter as it lacks the corroborating evidence of historical remnants that enabled evolutionists to trace so many differentiation paths for eukaryotes. As a matter of fact, since their discovery by Tissier, bifidobacteria had long

been considered very similar to lactobacilli: almost a century after Tissier (1900), Poupard *et al.* (1973) and others proposed on the basis of the morphology of cells and colonies that bifidobacteria be considered phylogenetically related to *Actinomyces*. Of course, it is utterly inadequate to infer phylogenetic differentiation merely on the basis of a few different morphological traits, though it may be a starting point, a working hypothesis against which many other different criteria should be tested in order to reach something like a stochastic function of the stability of the differentiated state in time.

As a bold approximation, one might say that in bacteria the stability of the differentiated state in time must be inferred from the right combination of morphological, functional and genetically determined traits, and that the number of such traits should be as high as possible since the probability of a large number being random is small. Again, it should be considered that the discriminating value for a phylogenetic character is higher with the increasing conservation of its genetic basis. More simply, it may be stated that the more a trait is stable, the more it appears unchanged in a high number of different strains, and the higher is the probable length of its evolutionary life. Added to the general difficulty of bacterial evolution is the scarce development of the genetics of the Gram-positive bacteria. With reference to *Lactobacillus*, *Bifidobacterium*, *Leuconostoc* and related genera, this means very few characters are known to be genetically determined, which in turn means that our interference in the time function is even more questionable or that the stability of the differentiated state is even less proven.

Of the different criteria proposed to establish the differentiated state of the *Bifidobacterium*, some rely on the 'discrimination level' of a single trait while others try to accumulate many traits, each one not very discriminating in itself. An example of the first criterion is the study of the enzyme fructose-6-phosphate phosphoketolase (Scardovi and Trovatelli, 1965), whose discriminating value is supposed to be high since it is a key enzyme in the carbohydrate metabolism of the genus. Cellular extracts of *Bifidobacterium* isolates from a number of various habitats show the presence of the F6PPK activity, suggesting a high degree of conservation for this trait. However, it should be borne in mind that there is no convincing experimental proof of the genetic determination of this trait and, moreover, the genetic structure of the strains that do not show F6PPK activity is unknown.

Sgorbati and London (1982) examined transaldolase, an enzyme in carbohydrate metabolism, which is supposed to be very significant in bacterial evolution as it controls the source of energy for the bacterial cell. The double-diffusion method of Oucherlony and microcomplement fixation techniques were used to study the intercurrent immunological relationships between the transaldolases present in several *Bifidobacterium* species

against eight antitransaldolase sera from *B. angulatum*, *B. asteroides*, *B. cuniculi*, *B. globosum*, *B. minimum*, *B. infantis*, *B. suis* and *B. thermophilum*. Species were grouped and immunological distances were estimated on the basis of similarity of amino acid sequences among enzyme molecules in different species (Sgorbati and Scardovi, 1979; Sgorbati and London, 1982). The degree of conservation of the primary structure of the enzymes was employed to develop a taxonomic index and an evolution dendrogram (Figure 8.2) for the grouping of species according to their habitat: human, animal, insect and sewage. The dendrogram suggests that isolates from insects and sewage habitats are at an extreme position in the space defined by antisera reactivity, and this might be interpreted as evidence of a longer evolutionary history of the species as represented by the isolates considered.

Interferences from these results should be considered as of the recurrently additive type, since the trait used to collect experimental evidence, even if important, cannot be taken as representative of a taxonomic entity, much less of a stable differentiated state of a bacterium. For this to be inferred one must be convincingly informed of the stability of the character on a genetic basis (and possibly with additional evidence of a different kind as well): of course, the evidence proposed by Poupard *et al.* (1973) and by Holdeman *et al.* (1977), based on the quantity of acetic acid produced (in *Bifidobacterium* it is expected to be larger than that of lactic acid but many genetic and environmental factors could hinder the expected result) needs an even broader basis of investigation since the specific informative value of the trait about *Bifidobacterium* evolution is very scarce. The same considerations hold too for the findings of Kandler (1970), who on the basis of the murein structure of the cell wall suggests that bifidobacteria are more closely related to lactobacilli than to *Actinomyces*.

The lack of genetic information can partly be overcome by studying the nucleic acid structure, but the phenotypes corresponding to the base sequences are not known in bacteria from these genera and therefore the interferences resulting from information on nucleic acid structure are still weak. Even the nature of the evidence is sometimes not fully informative. For example, the relative proportion of GC bases: without clear-cut information of its genetic value, it should be considered as a tentative differentiation with a possibly ambiguous meaning. Fox *et al.* (1977) report studies of rRNA sequences taken as 'evolutionary markers' on the assumption that these sequences are extensively conserved during the course of evolution. Woese *et al.* (1978) divide prokaryotes into *Archaebacteria* (including the methane-producing bacteria) and *Eubacteria* (including the bacteria associated with human physiology and pathology), bifidobacteria being included in the family Actinomycetaceae. Yet it must be noted that conservation alone is not very informative on the

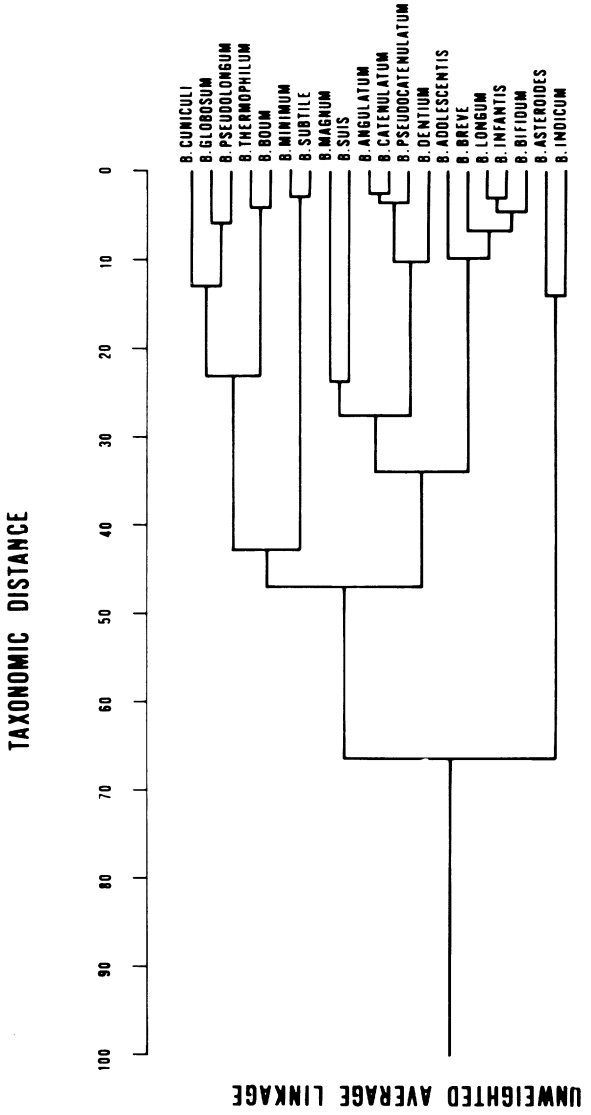

Figure 8.2 Dendrogram of the evalutionary relationships among the species of the genus *Bifidobacterium*.

evolutionary value of the trait studied unless the trait itself is shown to be subjected to selective pressure; otherwise, conservation may only mean not evolved.

8.3 The importance of the genus

The bifidobacteria that normally constitute part of the microflora in the human body are distributed in various niches: in the mouth, the ileum, the colon (where they are present in counts of 10^9/g and 10^{10}/g), the vagina and the cervix. Only the species *B. dentium* seems to cause dental caries, while all the other species, especially the intestinal ones, have a beneficial effect by maintaining a proper balance in the human intestinal flora. Given the therapeutic action of the intestinal bacterial group, both the food industry and pharmaceutical manufacturers have started adding cultures of bifidobacteria to milk and its by-products (yogurt in particular) and to pharmaceutical preparations (Rasìc and Kurmann, 1983; Bezkorovainy and Miller-Catchpole, 1989). The most common species used are *B. bifidum*, *B. longum* and *B. breve*, often combined with cultures of *Lactobacillus acidophilus* and *Streptococcus thermophilum* to facilitate acidification. These pharmaceutical preparations have been used in correcting the balance of intestinal microflora after antibiotic therapy for cirrhosis of the liver, in enterocolitis and in the case of intestinal stypsis.

The high levels of ammonia in the intestine, which enters the blood circulation in patients with portal systemic encephalopathy, can induce neurological disorders and the eventual development of coma (Podolsky and Isselbacher, 1987). Among the various therapies for the reduction of intestinal ammonia is a treatment which contains a strain of *B. bifidum* (Eugalanforte) or lactulose (Cephulac) (Kosman, 1976). The lactulose is not metabolized gastrically but in the colon, and it is degraded to lactic acid and acetic acid by the bifidobacteria, lactobacilli and streptococci. Lactulose has a beneficial effect for various reasons: it increases, albeit moderately, the growth of Gram-positive anaerobic bacteria, inhibits the growth of aerobic bacteria which are the major producers of ammonia, the ammonia produced in minor quantities lowers the pH and transforms itself into non-diffuse ammonia and therefore it is not absorbed by the intestine. The final result is that the acid produced lowers the pH, so transforming the ammonia to ammonium ion, which cannot diffuse through the intestinal wall into the bloodstream, with a consequent clinical improvement in the patient.

A clinical application of bifidobacteria was studied by Sekine *et al.* (1985): they injected 10^9 bifidobacteria (*B. infantis*) into a Meth-A sarcomatous tumour that had been subcutaneously transplanted into a BALB/c mouse and, after five injections, noticed a regression in tumour

mass. To determine which components of the cellular bacteria had an antitumoural action, three types of cell-wall preparations were then tested: whole peptidoglycan (WPG), cell-wall skeleton (CSW) and sonicated peptidoglycan (WPG). The impact of tumour regression after five injections was 70% (WPG), 40% (sonicated WPG) and 20% (CWS). From these results it can be inferred that it is the integrity of the cell wall (peptidoglycan in particular) that produces an antineoplastic action (Old *et al.*, 1959). These findings, recorded in experiments on mice only and not on man, suggest that bifidobacteria act like 'immunomodulators' that stimulate the reticuloendothelial system and activate natural killer cells, thereby strengthening the immunological defences against the neoplastic cells (Kohwi *et al.*, 1978).

8.4 List of species of the genus *Bifidobacterium*

The Approved Lists of Bacterial Names (Skerman *et al.*, 1980), which contain only the names of previously adequately described taxa, report 20 species under the genus *Bifidobacterium*. Another nine species have since been described and their names validated either because published in the *International Journal of Systematic Bacteriology* or validated under the procedure adopted in 1977 (Lessel, 1977). The following list reports the main information concerning each species together with the strains available at the ATCC (American Type Culture Collection), DSM (Deutsche Sammlung von Mikroorganismen) and JCM (Japan Collection of Microorganisms). Since certain morphological traits are characteristic of many *Bifidobacterium* species, the morphologies in Figure 8.3 are useful for species differentiation. Those features of the genus shared by a large part of the various species are reported in the general description and certain peculiar characteristics are discussed in a subsequent section of this chapter.

1 *Bifidobacterium adolescentis* (Reuter, 1963). In 1963 Reuter proposed four biovars (*a*, *b*, *c*, *d*) based on serological reactions and differences in the fermentation of mannitol and sorbitol. A study of DNA–DNA homology by Scardovi *et al.* (1971) indicates there is often little genetic relatedness between strains having the characteristics of Reuter's biovar groups. For example, biovars *b* and *d* of *B. adolescentis*, i.e. those which do not ferment sorbitol, cannot be distinguished phenotypically from *B. dentium*. Difficulties in distinguishing *B. adolescentis* on the basis of phenotypical characteristics from other bifidobacteria isolated from the faeces of human adults are reported by Yaeshima *et al.* (1992a), who used DNA base compositions and DNA–DNA homologies to assign phenotypically similar strains to different species; the PAGE procedure can also

be employed as an alternative (Biavati *et al.*, 1982, 1986). The cells exhibit a morphology very similar to that of many other species of the genus (Figure 8.3(1)). Isolated from faeces of human adult, bovine rumen and sewage. Type strain: E194a = ATCC 15703 = DSM 20083 = JCM 1275. Other strains: ATCC 11146; ATCC 15704 = JCM 7042; ATCC 15705 = DSM 20086 = JCM 7046; ATCC 15706; DSM 20087 = JCM 1251.

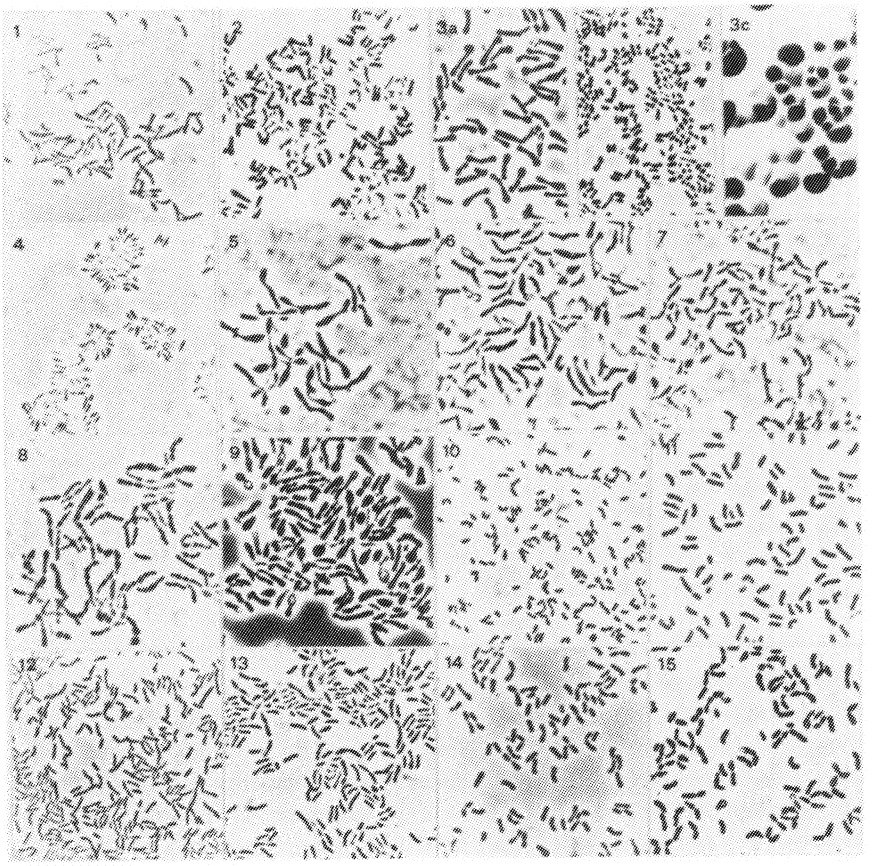

Figure 8.3 (cont'd on p. 292) Cellar morphology in the genus *Bifidobacterium*. Cells of the type strain were grown in anaerobic TPY medium stabs. Phase contrast photomicrographs × 825. (1) *B. adolescentis*; (2) *B. angulatum*; (3) *B. animalis* strain P23: a and b, from O and T colonies respectively; c, O and T colonies; (4) *B. asteroides*; (5) *B. bifidum*; (6) *B. boum*; (7) *B. breve*; (8) *B. catenulatum*; (9) *B. choerinum*; (10) *B. coryneforme*; (11) *B. cuniculi*; (12) *B. dentium*; (13) *B. gallicum*; (14) *B. gallinarum*; (15) *B. globosum*; (16) *B. indicum*; (17) *B. infantis*; (18) *B. longum*; (19) *B. magnum*; (20) *B. merycicum*; (21) *B. minimum*; (22) *B. pseudocatenulatum*; (23) *B. pseudolongum*; (24) *B. pullorum*; (25) *B. ruminantium*; (26) *B. saeculare*; (27) *B. subtile*; (28) *B. suis*; (29) *B. thermophilum*.

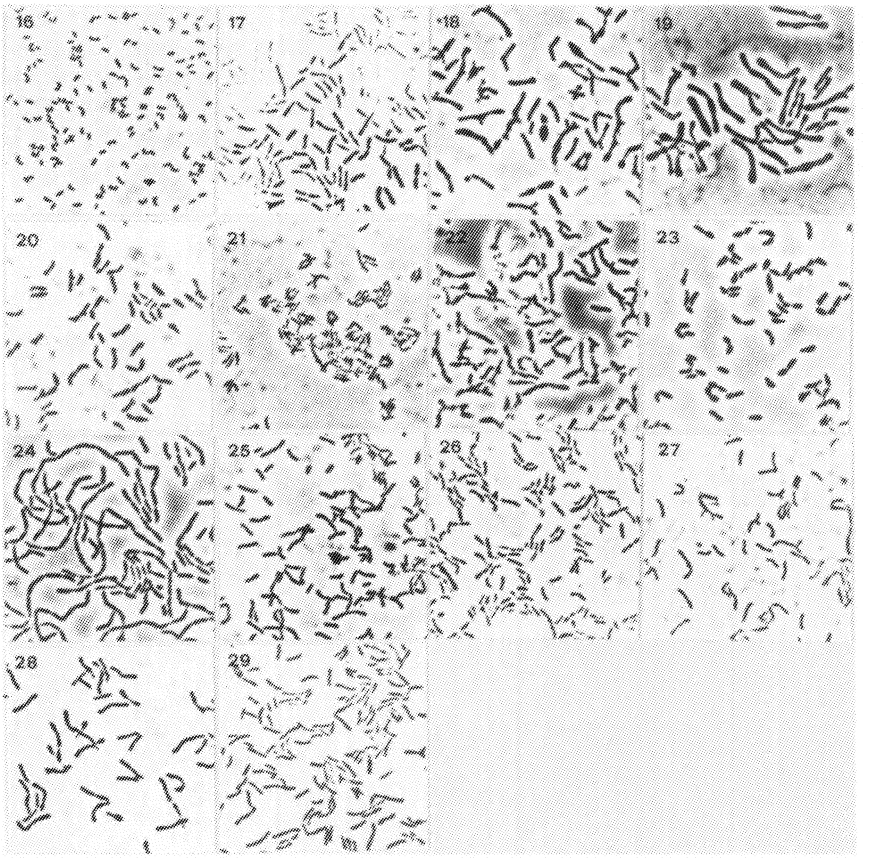

Figure 8.3 *cont'd*

2 *Bifidobacterium angulatum* (Scardovi and Crociani, 1974). This species was first isolated from human adults (only the type strain) and then found in sewage. Since no strains have been assigned to *B. angulatum* in many other studies concerning the presence of bifidobacteria in human faeces, the assumption that this species is a member of human intestinal microbiota is questionable. Its sensitivity to O_2 as measured by the depth of growth in stab is greater than that of most bifidobacteria; and CO_2 strongly enhances anaerobic growth. The morphology of cells grown in TPY agar stabs is unique among bifidobacteria (Figure 8.3(2)); characteristic are the arrangements of cells either in a V-shape or in palisade form similar to corynebacteria. Isolated from adult human faeces and sewage. Type strain: B 677 = ATCC 27535 = DSM 20098 = JCM 7096. Other strains. ATCC 27669; ATCC 27670 = DSM 20225 = JCM 1252; ATCC 27671.

3 *Bifidobacterium animalis* (Mitsuoka, 1969; Scardovi and Trovatelli, 1974). In 1969 Mitsuoka isolated from the faeces of various animals strains phenotypically very similar to *B. longum* and referred to as *B. longum* subsp. *animalis*; two biovars (*a*, *b*) were described. Two strains, one for each biovar, used as reference were found unrelated to *B. longum* upon DNA homology: one of them (biovar *b*) was genetically *B. pseudolongum* (Scardovi *et al.*, 1971) and the other (biovar *a*) was assigned to a group of isolates from animal faeces and sewage and proposed as *Bifidobacterium animalis* (Scardovi and Trovatelli, 1974). Recently, Biavati *et al.* (1992b) have identified the bifidobacteria from six samples of fermented milk products as *B. animalis* which, contrary to label information, was the only species of bifidobacteria present. It is generally held that bifidobacteria help to maintain a proper balance in human intestinal flora, and at the same time it is intuitively logical to assume that the bifidobacterial strains used as probiotic elements in the preparation of milk products should be from the human habitat. Strains of *B. animalis* isolated from commercial fermented milk products, faeces of rabbit, chicken and rat and from sewage have shown phase variations in colony appearance and in cellular morphology (Biavati *et al.*, 1992a). Some findings are documented in Figure 8.3(3a)–(3c). Most significant is the fact that the transition to colony morphotype transparent (T) and opaque (O) is accompanied by a dramatic change in cell dimension: minute and mostly spherical from T colonies while those from O colonies are large and show the species-specific shape, i.e. the central portion slightly enlarged. Isolated from faeces of calf, chicken, guinea pig, rabbit and rat; sewage; fermented milk products. Type strains: R 101–8 = ATCC 25527 = DSM 20104 = JCM 1190. Other strains: ATCC 27536 = DSM 20105 = JCM 1253; ATCC 27672; ATCC 27673; ATCC 27674 = JCM 7117.

4 *Bifidobacterium asteroides* (Scardovi and Trovatelli, 1969). Synonym: *Bacillus constellatus*. *Bifidobacterium asteroides*, one of three species isolated from honey bee gut, is the only one found in the intestine of *Apis mellifera* irrespective of geographical provenance (Scardovi and Trovatelli, 1969). Although unknown at present, the significance and the origin of bifidobacteria in the honey bee gut would make an interesting chapter of insect microbiology if they were found elsewhere in this class of animals. Cells grown anaerobically in fresh rich media are 2–2.5 μm long, pear-shaped or slightly curved, tend to have pointed ends and are usually arranged radially around a mass of common hold-fast material (Figure 8.3(4)). Colonies are circular, smooth and convex and feature a uniformly entire edge and glistening surface; their density is such that a colony can be removed by needle and can hardly be dispersed in water. Growth in static fluid culture tends to adhere to glass walls and to leave the liquid clear. A study of the presence of isozymes of transaldolase and 6-phosphogluconate

dehydrogenase (6-PGD) in 85 strains detected eight transaldolases and nine 6-PGDs (Scardovi *et al.*, 1979a). Of the 224 strains tested for the presence of plasmids 33% contained a large variety of extra chromosomal elements of varying molecular weight (Sgorbati *et al.*, 1982). Preliminary data on the structural relatedness among plasmids were collected by means of blot hybridization using many selected unrestricted plasmic probes. Thirteen plasmids were found in *B. asteroides* and their frequencies and distribution reported (Sgorbati *et al.*, 1986), although the functions coded are still unknown. Isolated from the hind-gut of western honey bees, occasionally found in *Apis cerana*, an Asiatic honey bee. Type strain: C51 = ATCC 25910 = DSM 20089. Other strain: ATCC 25909 = DSM 20431.

5 *Bifidobacterium bifidum* (Tissier, 1900; Orla-Jensen, 1924). Synonyms: *Bacillus bifidus communis*, *Bacillus bifidus*, *Bacteroides bifidus*, *Bacterium bifidum*, *Tissieria bifida*, *Nocardia bifida*, *Actinomyces bifidus*, *Actinobacterium bifidum*, *Lactobacillus bifidus*, *Lactobacillus parabifidus*, *Cohnistreptotrix bifidus*. Type species of the genus. Strains of this species have most often been used for the production of foods containing bifidobacteria, such as cultured milk and other dairy products, and in many therapeutic preparations for the treatment of digestive disturbances in infants that are not breast fed, of enterocolitis, constipation, cirrhosis of the liver, imbalance of intestinal flora following antibiotic therapy and for the promotion of intestinal peristalsis. Some cells grown in TPY agar stabs have an 'amphora-like' cell shape (Figure 8.3(5)). They are anaerobic, dying rapidly in aerobic subcultures. *Bifidobacterium bifidum* is among the most easily recognized species in the genus because of its characteristic fermentation pattern. Isolated from faeces of human adults and infants, suckling calf and human vagina. Type strain: TI = ATCC 29521 = DSM 20456 = JMC 1255. Other strains: ATCC 11863 = DSM 20239 = JMC 1209; ATCC 15696 = JCM 7004; ATCC 35914; DSM 20082 = JCM 1254; DSM 20215.

6 *Bifidobacterium boum* (Scardovi *et al.*, 1979b). From a large number of animal strains surveyed by DNA–DNA hybridization, 36 strains from the rumen and five from piglet faeces were assigned to the new species *B. boum* (Scardovi *et al.*, 1979b). Two other species found in the same habitat, *B. choerinum* and *B. thermophilum*, cannot be distinguished on common phenotypical grounds, the differences in their morphology can be used for their distinction (Figure 8.3(6)) as can transaldolase electrophoresis (Scardovi *et al.*, 1979a) and PAGE protein electrophoresis (Biavati *et al.*, 1982), which also permit clear-cut identification. Isolated from bovine rumen, piglet faeces. Type strain: Ru 917 = ATCC 27917 = DSM 20432 = JCM 1211. No other strains available in culture collections.

7 *Bifidobacterium breve* (Reuter, 1963). Synonym. *Bifidobacterium parvulorum*. Cells are short, slender or thick and with or without

bifurcations, a morphology that suggests the specific epithet (Figure 8.3(7)). The two biovars *a* and *b* were identified by Reuter (1963), and serologically related strains were referred to a separate species, i.e. *B. parvulorum*. DNA homology tests on type strains and other strains previously designated as *B. breve* and *B. parvulorum* proved the genetic identity of Reuter's two species (Scardovi *et al.*, 1971). Isolated from the faeces of infant and suckling calf; human vagina; sewage. Type strains: S1 = ATCC 15700 = DSM 20213 = JCM 1192. Other strains: ATCC 11147; ATCC 15698 = DSM 20091 = JCM 1273; ATCC 15701 = JCM 7016.

8 *Bifidobacterium catenulatum* (Scardovi and Crociani, 1974). This species is reported as one of the most frequently isolated from adult and infant faeces (Biavati *et al.*, 1982, 1986). Lauer and Kandler (1983), in a study on DNA–DNA homology, murein types and enzyme patterns of the type strains of the genus *Bifidobacterium*, suggest that *B. catenulatum* and *B. pseudocatenulatum* may be subspecies of *B. catenulatum*, i.e. *B. catenulatum* subsp. *catenulatum* and *B. catenulatum* subsp. *pseudocatenulatum*. The same suggestion is posited by Biavati *et al.* (1982) concerning the use of a PAGE procedure to distinguish genetically related and unrelated species of bifidobacteria. However, the G+C content of their DNA differs by 3 mol%, and the GC% of *B. catenulatum* is the lowest value found in bifidobacteria (see Table 8.2); none of the strains genetically identified as *B. catenulatum* ferments starch or mannose, whereas strains of *B. pseudocatenulatum* ferment these compounds. The isozymes of transaldolase and 6PGD of the two species migrated very differently (see Table 8.2); furthermore *B. catenulatum* as yet has never been isolated from a faeces of suckling calves. Cells grown in TPY agar stabs are generally arranged in chains of three or more globular elements (Figure 8.3(8)). Isolated from faeces of infant and human adult; human vagina; sewage. Type strain: B669 = ATCC 27539 = DSM 20103 = JCM 1194. Other strains: ATCC 27675; ATCC 27676; ATCC 27677; DSM 20224 = JCM 7130.

9 *Bifidobacterium choerinum* (Scardovi *et al.*, 1979b). *Bifidobacterium choerinum* exhibits a sugar fermentation pattern very similar to that of *B. thermophilum* and *B. boum*, two other species frequently found in piglet faeces. These three species can be distinguished by PAGE patterns of soluble proteins or by transaldolase electrophoresis. Cells grown in TPY agar stabs show a morphology similar to that of *B. globosum* (Figure 8.3(9)). Isolated from faeces of piglet and sewage. Type strain: SU 806 = ATCC 27686 = DSM 20434. No other strains available in culture collections.

10 *Bifidobacterium coryneforme* (Scardovi and Trovatelli, 1969; Biavati *et al.*, 1982). *Bifidobacterium coryneforme* was inadvertently omitted from the Approved Lists (Skerman *et al.*, 1980) and is therefore without

taxonomic standing. Biavati *et al.* (1982) proposed reinstatement of the name *B. coryneforme* on the basis of its different electrophoretic pattern of cellular proteins and the previously confirmed differential characters. The growth is more abundant in MRS than in PYG. Cells grown in MRS agar stabs very rarely form radial groupings of cells. Their morphological traits are shown in Figure 8.3(10). Isolated from the intestine of honey bee, *Apis mellifera* subsp. *mellifera* and subsp. *caucasica*. Type strain: C 215 = ATCC 25911 = DSM 20216 = JCM 5819. No other strains available in culture collections.

11 *Bifidobacterium cuniculi* (Scardovi *et al.*, 1979). *Bifidobacterium cuniculi* inhabits rabbit intestinal tracts and its morphology (Figure 8.3(11)) is similar to that of *B. globosum*, *B. pseudocatenulatum* and *B. animalis*, which are also found in the same habitat. The fermentation patterns can be used to distinguish this species from the other three because unlike the latter, *B. cuniculi* does not ferment lactose, ribose or raffinose. *Bifidobacterium magnum*, another species isolated from the same source, can be distinguished via morphology (Figure 8.3(19)), in addition to its different fermentation pattern. Isolated from faeces of rabbit. Type strain: Ra 93 = ATCC 27916 = DSM 20435. No other strains available in culture collections.

12 *Bifidobacterium dentium* (Scardovi and Crociani, 1974). Synonyms: *Bifidobacterium appendicitis*, *Actinomyces eriksonii*, *Bifidobacterium eriksonii*. Scardovi *et al.* (1971), in a DNA–DNA homology study, first identified bifid strains isolated from human dental caries, faeces of human adult and human vagina as a distinct group from other strains, also identifiable phenotypically as *B. adolescentis*, and suggested considering them as 'dentium' group distinct from *B. adolescentis*. The phenotypic distinction from biovars *b* and *d* of *B. adolescentis* and *B. dentium* should be based either on transaldolase isozymes (Scardovi *et al.*, 1979a) or on electrophoretic patterns of soluble cellular proteins (Biavati *et al.*, 1982). The morphology of these two species is quite similar (Figure 8.3(1) and (12)). *Bifidobacterium dentium*, as well as *B. angulatum* and *B. catenulatum*, requires riboflavin and pantothenate for growth. That this species is frequently isolated from dental caries and often found in human clinical samples, supports the view that its definition by Georg *et al.* (1965) as a pathogenic anaerobic species may be appropriate. Isolated from human dental caries and oral cavities; faeces of human adult, human vagina, human clinical samples. Type strain: B 764 = ATCC 27534 = DSM 20436 = JCM 1195. Other strains: ATCC 15423 = DSM 20084; ATCC 15424; ATCC 27678; ATCC 27679; ATCC 27680; DSM 20221 = JCM 7135.

13 *Bifidobacterium gallicum* (Lauer, 1990). The description of this species is based on phenotypic and genotypic characterization of a strain found by

H. Beerens (Institut Pasteur de Lille, France) in 1967 as a human faecal isolate. This isolate proved to have very low genetic relatedness to the other species of the genus and to contain a type of peptidoglycan unique among the previously described species. Provided that the characteristics of the monotype strain are confirmed in further isolates, the identification of *B. gallicum* is possible from its carbohydrate fermentations: pentoses and starch are fermented whereas lactose, melibiose and raffinose are not. *Bifidobacterium gallicum* has been isolated from human faeces but its true habitat remains uncertain because no additional strains have been isolated by researchers in intestinal ecology. Its morphological appearance is shown in Figure 8.3(13). Monotype strain P 6 = DSM 20093.

14 *Bifidobacterium gallinarum* (Watabe *et al.*, 1983). This species, described by Watabe *et al.* (1983) as having very low or no genetic relatedness with the previously described species of the genus *Bifidobacterium*, was subsequently found to be closely related (61%) to a new species, *B. saeculare*, described by Biavati *et al.* (1991a). Carbohydrate fermentation patterns are characterized by the ability of *B. gallinarum* to ferment inulin and dextrin, which enables this species to be distinguished from the other starch-negative bifidobacteria. It has been reported (Watabe *et al.*, 1983) that there is a strain – Barnes EBF 92/68 – in this species that failed to ferment glucose; this is the only case known previously of the bifidobacteria strain that does not ferment glucose. The cell morphology is shown in Figure 8.3(14). Isolated from chicken caeca. Type strain: Ch 206–5 = ATCC 33777 = DSM 20670. Other strains ATCC 33778 = JCM 6291.

15 *Bifidobacterium globosum* (Scardovi *et al.*, 1969; Biavati *et al.*, 1982). This species was considered as a synonym of *B. pseudolongum* by Rogosa (1974) and not reported in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980). These two species show about 70% DNA–DNA homology and have similar fermentation patterns and morphology (Figure 8.3(15) and (23)). However, the reported G+C contents of their DNA differ by 3 mol% (Scardovi *et al.*, 1971). The classification as an independent species is also sustained by differences in 6-phosphogluconate dehydrogenase (6PGD) zymogram, by their DNA similarity to other related species and by the presence of plasmids of larger molecular weight found only in *B. globosum* (Sgorbati *et al.*, 1982). Further indications on the validity of the species *B. globosum*, the revival of which was proposed by Biavati *et al.* (1982), include the identical PAGE protein patterns of selected strains from various sources, which are quite distinct from those of the most closely related species, *B. pseudolongum*. Recently, Yaeshima *et al.* (1992b), in a study comparing the DNA base composition, DNA similarities and phenotypic characteristics of *B. pseudolongum* and *B. globosum* strains, have proposed that *B. pseudolongum* and *B. globosum*

should be unified into two subspecies: *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum*. *Bifidobacterium globosum* is undoubtedly the predominant bifidal species in a large number of animal digestive tracts. Isolated from faeces of piglet, suckling calf, rat, rabbit and lambs; sewage; bovine rumen and in a single specimen of human infant faeces. Type strain: Ru 224 = ATCC 25865 = DSM 20092 = JCM 5820. Other strain: ATCC 25864.

16 *Bifidobacterium indicum* (Scardovi and Trovatelli, 1969). *Bifidobacterium indicum*, as well as *B. asteroides* and *B. coryneforme*, are the bifids inhabiting the intestine of honey bees. Cells grown in TPY agar stabs are short, generally paired and coccoid; star-like clusters are not observed (Figure 8.3(16)). Colonies are less dense than those of *B. asteroides*. In liquid media the cells do not adhere to the walls and have an even turbidity and dispersible sediment. One-hundred and twenty-two strains were examined for their transaldolase and 6PGD zymograms; different isozymes obtained by starch gel electrophoresis were reported (Scardovi *et al.*, 1979a). Sixty-seven percent of the 106 strains of *B. indicum* surveyed for the presence of plasmids were positive (Sgorbati *et al.*, 1982). The structural relatedness among plasmids obtained by means of blot hybridization using selected unrestricted plasmid probes points to the presence of three differing plasmids (Sgorbati *et al.*, 1986). Unrelated by DNA homology to any other species of the genus. Isolated from intestine of *Apis cerana* and *Apis dorsata*. Type strain: C 410 = ATCC 25912 = DSM 20214 = JCM 1302. Other strain: ATCC 25913.

17 *Bifidobacterium infantis* (Reuter, 1963). Synonyms: *Bididobacterium liberorum*, *Bifidobacterium lactentis*, *Actinomyces parabifidus*. Cellular morphology is similar to that of many other species of the genus (Figure 8.3(17)). Most pertinent literature indicates that this species is predominant in the faeces of breast-fed infants and is apparently host-specific given that no strains of *B. infantis* have been found in the faeces of human adults (Biavati *et al.*, 1982, 1986). The close genetic relationships between *B. infantis* and *B. longum* are reflected in the similar fermentation activity and electrophoretic patterns of their soluble cellular proteins. Isozyme patterns of transaldolase and 6PGD were determined in 63 and 12 strains which were allotted via DNA homology to *B. infantis* and *B. longum*, respectively; 90% of *B. infantis* strains had a more anodal transaldolase (isozyme 5, migration 100) than that possessed by 72% of *B. longum* strains (isozyme 8, migration 90) (Scardovi *et al.*, 1979a). Identification of strains belonging to one of the two species remains a difficult task. The rules suggested by Scardovi (1986) can be adopted: strains not fermenting arabinose should be held as *B. infantis* while strains fermenting both arabinose and melezitose should regarded as *B. longum*; strains fermenting arabinose (and xylose) but not melezitose are *B. infantis* if their

transaldolase migrates more anodically (isozyme 5, migration 100), and *B. longum* if their isozyme is less anodal (isozyme 8, migration 90). Isolated from faeces of infant and suckling calk; human vagina. Type strain: S12 = ATCC 15697 = DSM 20088 = JCM 1222. Other strains: ATCC 15702 = DSM 20090 = JCM 1272; ATCC 17930 = DSM 20218 = JCM 1260; ATCC 25962 = DSM 20223 = JCM 1210; ATCC 27920.

18 *Bifidobacterium longum* (Reuter, 1963). The cellular morphology of strains belonging to this species is characterized by very elongated and relatively thin cells with rare branchings (Figure 8.3(18)). *Bifidobacterium longum* may be considered the most common species of bifidobacteria, being found both in infant and adult faeces (Biavati *et al.*, 1984, 1986). Accordingly, the importance of this species in the production of food containing bifidobacteria such as cultured milk, other milk products or added preparations made by pharmaceutical industries for therapeutic purposes is obvious. Methods for the proper identification of the bifidobacteria employed in fermented milks are reported by Biavati *et al.* (1992b). Reuter (1963), in the description of the species, points out the presence of biovar *a*, which slowly ferments mannose in human adults, and biovar *b*, which is mannose-negative in infants. Mitsuoka (1969) proposed the *B. longum* subsp. *animalis* biovars *a* and *b*, which were subsequently recognized on the basis of DNA homology as *B. animalis* and *B. pseudolongum*, respectively (Scardovi *et al.*, 1971; Scardovi and Trovatelli, 1974). The DNA of this species is 50–76% related to that of *B. infantis*; comments about identification problems are reported under *B. infantis*. Furthermore, a group of 180 strains isolated form the faeces of suckling calves could not be identified because of their high degree (over 80%) of DNA homology with both *B. infantis* and *B. longum*, and hence could conceivably constitute a 'continuum' between the two species (Scardovi *et al.*, 1979a). *Bifidobacterium longum* is apparently the only species among those usually found in human faeces which possess a large variety of plasmids (Sgorbati *et al.*, 1982). *Bifidobacterium infantis* does not carry plasmids, although strains of both species, generally isolated from the same species, have been studied; the same was true for the 45 strains studied as *B. infantis–B. longum* 'intermediate' found in calf faeces. Isolated from faeces of human adult, infant and suckling calves; human vagina; sewage. Type strain: E 194b = ATCC 15707 = DSM 20219 = JCM 1217. Other strains: ATCC 15708 = JCM 7054.

19 *Bifidobacterium magnum* (Scardovi and Zani, 1974). This species is characterized by the unusually large dimension of its cell, especially when grown in the absence of Tween 80. Cells grown in TPY agar are 2 μm in diameter, 10–20 μm in length and are frequently arranged in aggregates (Figure 8.3(19)). Other features of the species are: the high stimulative effect of Tween 80, the optimum pH for growth (5.3–5.5), which

characterize this species as the only acidophilic one within the genus, the unique isozyme pattern among bifidobacteria (Scardovi *et al.*, 1979a) and the PAGE protein pattern (Biavati *et al.*, 1982). Isolated from rabbit faeces. Type strain: RA 3 = ATCC 27540 = DSM 20222 = JCM 1218. Other strains: ATCC 27681 = JCM 7120; ATCC 27682 = DSM 20220 = JCM 7132.

20 *Bifidobacterium merycicum* (Biavati and Mattarelli, 1991). Four strains among several hundred bifidobacteria isolated from bovine rumen were recognized primarily on the basis of DNA–DNA hybridization results as members of a new homology group and posited as a new species. The morphological traits of the strains belonging to *B. merycicum* are not as distinctive as those of many other bifidobacteria (Figure 8.3(20)). This species is more sensitive to oxygen than most bifidobacteria; its fermentation characteristics, when compared to those of *Bifidobacterium* species that have been isolated from bovine rumen, may be helpful in identification (Biavati and Mattarelli, 1991). Isolated from bovine rumen. Type strain: Ru 915B = ATCC 49391 = DSM 6492 = JCM 8219. Other strains: DSM 6493 = JCM 8220; DSM 6494.

21 *Bifidobacterium minimum* (Scardovi and Trovatelli, 1974; Biavati *et al.*, 1982). The specific epithet refers to the very small size of the cells, a morphology resembling that of *B. asteroides* without star-like aggregates (Figure 8–3(21)). In addition to morphology, this species possesses two other features that are unique among bifidobacteria: the lys-ser murein type (Lauer and Kandler, 1983) and the last anodal isozyme of transaldolase (Scardovi *et al.*, 1979a). This taxon was called a 'minimum' DNA homology group by Scardovi and Trovatelli (1974) and consists of only two strains. Isolated from sewage. Type strain: F 392 = ATCC 27538 = DSM 20102 = JCM 5821. No other strains in culture collections.

22 *Bifidobacterium pseudocatenulatum* (Scardovi *et al.*, 1979b). According to Biavati *et al.* (1986), this species is the one most frequently found in human adult faeces. This fact may be influenced by difficulties in differentiating *B. pseudocatenulatum* from *B. catenulatum* (for comments on identification problems, see *B. catenulatum*); in any case both species are very common members of the intestinal microbiota. Strain-to-strain variability in cell morphology is common in this species. The morphological traits of the type strains grown on TPY agar stabs are shown in Figure 8.3(22). Riboflavin, pantothenate and nicotinic acid required for growth. The DNA of this species is closely related to that of *B. catenulatum* and practically unrelated to that of any other species of the genus. Isolated from faeces of human adult, infant and suckling calf and sewage. Type strain: B 1279 = ATCC 27919 = DSM 20438 = JCM 1200. Other strain: DSM 20439.

23 *Bifidobacterium pseudolongum* (Mitsuoka, 1969). Bifid strains isolated from a variety of animals were recognized as *B. pseudolongum* and the four biovars *a*, *b*, *c* and *d* were proposed on the basis of differences in the fermentation of mannose, lactose, cellobiose and melezitose (Mitsuoka, 1969). The strain Mo-2-10 representative of biovar *d*, was found completely homologous to strain C10-46 of *B. longum* subsp. *animalis* biotype *b* (Scardovi *et al.*, 1971). *Bifidobacterium pseudolongum* has about 70% DNA–DNA homology with *B. globosum*. Moreover, these two species are phenotypically very similar having the same murein type (Lauer and Kandler, 1983) and identical morphology (Figure 8.3(15) and (23)). They have recently been proposed as subspecies: *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum* (Yaeshima *et al.*, 1992b). Further comments on the differentiation of *B. globosum* and *B. pseudolongum* are reported under *B. globosum*. Isolated from faeces of pig, chicken, dog, bull, calf, rat and guinea pig. Type strain: PNC-2-9G = ATCC 25526 = DSM 20099 = JCM 1205. Other strains: JCM 1264; DSM 20094 = JCM 1266; DSM 20095 = JCM 1267.

24 *Bifidobacterium pullorum* (Trovatelli *et al.*, 1974). Morphology is of help in recognizing this species, whose cells are mostly arranged in irregular chains often of great length (Figure 8.3(24)). For good growth, nicotinic acid, pyridoxin, thiamin, folic acid, *p*-aminobenzoic acid and Tween 80 are required. The G+C content of the DNA is 67 mol% (as determined by thermal denaturation) the highest value reported for bifidobacteria. Unlike the other species of the genus, the isomeric type of lactic acid formed is DL. Isolated from chicken faeces. Type strain: P 145 = ATCC 27685 = DSM 20433. No other strains available in culture collections.

25 *Bifidobacterium ruminantium* (Biavati and Mattarelli, 1991). Bifidobacteria are present in rumen in large numbers, especially when animals are fed starch-rich diets. The species commonly found in this habitat are *B. globosum*, *B. thermophilum* and *B. boum*. Two other species, *B. ruminantium* and *B. merycicum*, have recently been described as inhabitants of cattle rumen; they have similar morphology (Figure 8.3(20) and (25)) but very low levels of DNA relatedness. The fermentation characteristics distinguishing the *Bifidobacterium* species isolated from bovine rumen are reported by Biavati and Mattarelli (1991). *Bifidobacterium ruminantium* DNA is 54% homologous (average value) to the DNA of *B. adolescentis*. Additional experiments (electrophoretograms of soluble cellular proteins and electrophoretic patterns of isozymes), performed to verify the taxonomic position of these two species confirmed that they should be recognized as distinct. *Bifidobacterium ruminantium* is more sensitive to oxygen than most bifidobacteria, carbon dioxide did not affect this sensitivity but it strongly enhanced anaerobic growth. Isolated from rumen

of cattle. Type strain: Ru 687 = ATCC 49390 = DSM 6489 = JCM 8222. Other strains: DSM 6490 = JCM 8509; DSM 6491.

26 *Bifidobacterium saeculare* (Biavati *et al.*, 1991a). Trovatelli *et al.* (1974) found that four strains of bifidobacteria isolated from faeces of rabbit were characterized as 'unassigned homology group I'. Subsequently, on the basis of DNA–DNA hybridization, phenotypic characters and electrophoretic patterns of proteins and of isozymes the 'unassigned homology group I' was described as a new species and named *B. saeculare*. The specific epithet is in honour of the Ninth Centenary of the Foundation of Bologna University. A 60–64% range of intraspecific DNA homologies was observed between *B. saeculare* and both *B. pullorum* and *B. gallinarum*, two species isolated from chicken faeces. Additional experiments on fermentation analysis (19 complex carbohydrates and mucins were tested), isozyme electrophoresis and penicillin-binding protein detection were performed to verify the taxonomic position of *B. saeculare* with respect to *B. pullorum* and *B. gallinarum*. The various characteristics distinguishing the three species are listed in a table by Biavati and Mattarelli (1991). The PAGE procedure can be used alternatively. Cells grown in TPY agar stabs are short, slightly curved rods, isolated or in pairs (Figure 8.3(26)). Isolated from rabbit faeces. Type strain: Ra 161 = ATCC 49392 = DSM 6531 = JCM 8508. Other strains: DSM 6532; DSM 6533.

27 *Bifidobacterium subtile* (Biavati *et al.*, 1982). This taxon was referred by Scardovi and Trovatelli (1974) as a 'subtile' DNA homology group. Its cell morphology (Figure 8.3(27)) is similar to that of *B. breve* (Figure 8.3(7)), although the cells of the latter are usually shorter and thicker, swollen and branched. The two species have very similar fermentation patterns although *B. subtile* ferments starch and gluconate whereas *B. breve* does not and lactose is fermented only by *B. breve*. Its DNA is not related to that of any of the other species. The optimum temperature for growth (34–35°C) is the lowest among bifidobacteria (37–41°C range). Isolated from sewage. Type strain: F 395 = ATCC 27537 = DSM 20096 = JCM 5822. Other strains: ATCC 27683 = JCM 7109; ATCC 27684.

28 *Bifidobacterium suis* (Matteuzzi *et al.*, 1971). *Bifidobacterium suis*, commonly found in the faeces of piglets, share this habitat with other bifid species, i.e. *B. globosum*, *B. pseudolongum*, *B. thermophilum*, *B. boum* and *B. choerinum*, and can be distinguished from the latter species on the basis of fermentation characters: *B. globosum* and *B. pseudolongum* ferment starch whereas *B. suis* does not, and the last three species do not ferment arabinose and xylose whereas *B. suis* does. Cells are slender, elongated, with rare terminal bifurcations or clumps (Figure 8.3(28)). Riboflavin is the only demonstrable vitamin required for growth. Most strains posses a strong ureolytic activity. High levels of urease activity were

present in the cells grown in the absence of urea, suggesting that this enzyme is not inducible (Crociani and Matteuzzi, 1982). Matteuzzi *et al.* (1971) did not find any DNA–DNA relationship between their reference strain of *B. suis* and any strain of the other species tested. In a subsequent study of DNA–DNA homology in the type strains of the genus *Bifidobacterium* (Lauer and Kandler, 1983) it was found that *B. suis* and *B. longum* are genetically closely related to each other (70% DNA–DNA homology) and to *B. infantis* (about 65% DNA–DNA homology). Isolated from piglet faeces. Type strain: Su 859 = ATCC 27533 = DSM 20211 = JCM 1269. Other strains: ATCC 27531; ATCC 27532 = JCM 7139.

29 *Bifidobacterium thermophilum* (Mitsuoka, 1969). Synonym: *Bifidobacterium ruminale*. Mitsuoka applied this specific epithet because of the ability of the strains belonging to this species to grow at 46.5°C. Cells are slender, slightly curved, often with tapered ends, protuberances or irregularities near the junction of paired cells, rare branchings and arranged singly or in pairs never in clumps or in angular disposition (Figure 8.3(29)). The four biovars *a*, *b*, *c* and *d* were distinguished by Mitsuoka (1969) according to differences in the fermentation of melezitose and lactose. For comments on the identification of *B. thermophilum*, *B. boum* and *B. choerinum* see *B. choerinum*. Isolated from faeces of pig, chicken and suckling calf; bovine rumen; sewage. Type strain: P2–91 = ATCC 25525 = DSM 20210 = JCM 1207. Other strains: ATCC 25866 = DSM 20212 = JCM 1268; ATCC 25867; DSM 20209.

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